Neutrophil A<sub>2A</sub> Adenosine Receptor Inhibits Inflammation in a Rat Model of Meningitis: Synergy with the Type IV Phosphodiesterase Inhibitor, Rolipram

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Bacterial meningitis is a disease worsened by neutrophil-induced damage in the subarachnoid space. In this study, the A<sub>2A</sub> adenosine receptors on human neutrophils were characterized, and the role of A<sub>2A</sub> receptors on the trafficking of leukocytes to the cerebrospinal fluid and on blood-brain barrier permeability (BBBP) was assessed in a rat meningitis model. Neutrophils bind the A<sub>2A</sub> selective antagonist, <sup>125</sup>I-ZM241385 (B<sub>max</sub> = 843 receptors/neutrophil; K<sub>D</sub> = 0.125 nM). A selective A<sub>2A</sub> receptor agonist, WRC-0470 (2-cyclohexylmethylidene-ahydrazoneadenosine; 0.03–1 μM), alone and synergistically with the type IV phosphodiesterase inhibitor, rolipram, increased neutrophil [cAMP], and reduced cytokine-enhanced neutrophil adherence, superoxide release, and degranulation. These effects of WRC-0470 were reversed by ZM241385 (100 nM). In a lipopolysaccharide-induced rat meningitis model, WRC-0470 (0–0.9 μg/kg/h), with or without rolipram (0–0.01 μg/kg/h), inhibited pleocytosis and reduced the lipopolysaccharide-induced increase in BBBP, indicative of decreased neutrophil-induced damage.

Four subtypes of 7-transmembrane G-coupled adenosine receptors have been cloned from mammalian cells (A<sub>1</sub>, A<sub>2A</sub>, A<sub>3B</sub>, and A<sub>3</sub>) [1]. All four subtypes have been detected on leukocytes, with variable expression and function depending on cell type and species [2]. Adenosine is a regulator of inflammatory responses. There is evidence of functional A<sub>1</sub> and A<sub>2A</sub> receptors on polymorphonuclear leukocytes (PMNL) [3–5]. By binding to adenosine receptors on PMNL, adenosine can have variable and even opposing effects on function, depending upon the receptor subtype(s) activated.

When adenosine or selective synthetic agonists bind to A<sub>1</sub> receptors, PMNL adherence to endothelium is increased [6]. In addition, chemotaxis [7] and oxidative activity in response to immune complexes are increased [8]. Conversely, the binding of adenosine or selective agonists to A<sub>2A</sub> adenosine receptors increases PMNL [cAMP], decreases the adherence of stimulated PMNL to endothelium [6], reduces the release of reactive oxygen species [3, 8–16], restores cytokine-inhibited PMNL migration [17], and decreases the degranulation of primary lysosomes [18–21]. These effects of A<sub>2A</sub> adenosine receptors are antiinflammatory.

Type IV phosphodiesterase (PDE) inhibitors also have antiinflammatory effects [14, 22, 23]. Type IV is the predominant isozyme of PDE found in PMNL [24]. There is disagreement in the literature concerning the ability of type IV PDE inhibitors to synergize with adenosine A<sub>2A</sub> receptor agonists to decrease stimulated PMNL oxidative activity. Cronstein et al. [25] failed to see synergy between S<sup>5</sup>-ethylcarboxamidoadenosine and the type IV PDE inhibitor, Ro-201724, to decrease FMLP-stimulated PMNL oxidative activity. In contrast, we observed marked synergy between the type IV PDE inhibitor, rolipram, and a selective adenosine A<sub>2A</sub> receptor agonist to reduce the PMNL oxidative burst [14].

Agonists of A<sub>2A</sub> adenosine receptors or inhibitors of type IV PDE may have therapeutic utility as antiinflammatory agents. However, high concentrations of adenosine receptor agonists and type IV PDE inhibitors can have unacceptable side effects in vivo. We hypothesized that it would be possible to use a selective A<sub>2A</sub> adenosine receptor agonist in combination with a type IV PDE inhibitor to inhibit in vivo PMNL-mediated inflammatory responses at doses below those that cause adverse side effects, such as hemodynamic activities from adenosine agonists [26] or emesis from PDE inhibitors [27].

Bacterial meningitis is a PMNL-mediated inflammatory disease of the central nervous system (CNS) that occurs when bacteria gain entry to the cerebrospinal fluid (CSF; reviewed in [28, 29]). In meningitis, bacteria release cell surface com-

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ponents into the CSF (e.g., lipopolysaccharide [LPS] and peptidoglycan) that trigger resident CNS cells to produce inflammatory cytokines, specifically interleukin-1 and tumor necrosis factor (TNF)-α. After these early events, the cytokines induce a number of cellular processes that culminate in the chemotraction of PMNL, adherence of PMNL to cerebrovascular endothelium, and subsequent pleocytosis of the PMNL into the CSF. Once the PMNL have entered the CSF, exposure to various stimuli induces release of products, including reactive oxygen and nitrogen species that contribute to the pathophysiology of bacterial meningitis [30, 31].

In the present study, we characterized adenosine A2A receptors on human PMNL with the high-affinity selective A2A radioligand, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)-3-[125I]iodophenol (125I-ZM241385) [32] and determined the effect of the selective A2A adenosine receptor agonist, 2-cychohexylmethylidenehydrazinoadenosine (WRC-0470) [33, 34], and the selective A2A antagonist, ZM241385, on human PMNL activity. We also investigated the role of cAMP in mediating the antiinflammatory effects of adenosine A2A receptor activation with or without PDE IV inhibition in in vitro assays of PMNL function. In a rat model of LPS-induced meningitis, we examined the effect of WRC-0470 on PMNL emigration into the CSF and on blood-brain barrier permeability (BBBP) in the presence and absence of rolipram.

Materials and Methods

Reagents

WRC-0470 [34] was supplied by Pauline Martin of Discovery Therapeutics (Richmond, VA). Stock solutions were prepared in dimethyl sulfoxide (DMSO; 10 mM) and stored at −20°C. WRC-0470 was selected from 20 2-substituted adenosine analogs for further evaluation on the basis of its affinity and selectivity for A2A receptors. As we reported elsewhere [34], the human adenosine receptor \( K_D \) values for WRC-0470 are as follows: \( A_{2A} \), 270 nM; \( A_1 \), 48 μM; \( A_{2B}, 430 \) μM; and \( A_3 \), 903 nM. ZM241385 was a gift from Simon Poucher (Zeneca Pharmaceuticals, Cheshire, UK). Stock solutions (1 and 10 mM in DMSO) were stored at −20°C. Recombinant human TNF-α was a gift from Dianippon Pharmaceutical (Osaka, Japan; specific activity, 600 pg/U). Stock solutions were made in Hank’s balanced salt solution (HBSS)-0.1% human serum albumin (HSA) at 2 × 10^5 U/mL, aliquoted into single-day samples, and frozen at −70°C. Rolipram [4-(3'-cyclopropyloxy-4'-methoxyphenyl)-2-pyrididone] was a gift of Berlex Laboratories (Cedar Knolls, NJ). Stock solutions (10 mM) were made in DMSO and stored at −70°C.

Other reagents. Neutrophil isolation medium (ficol-hyphaque) was purchased from ICN Biomedicals (Aurora, OH), Accurate Chemicals and Scientific (Westbury, NY), and Cardinal Associates (Santa Fe, NM). We purchased the following reagents from Sigma (St. Louis): LPS (derived from Escherichia coli 026:B6), human fibrinogen (fraction I, type 1, from human plasma), adenosine deaminase type X (ADA), cytochrome c (type IV from horse heart), superoxide dismutase (SOD; bovine liver), FMLP, lyophilized Micrococcus lysodeikticus, and catalase. Dihydrorhodamine 123 was purchased from Molecular Probes (Eugene, OR). FACS lysing solution was purchased from Becton Dickinson (San Jose, CA). Ketamine and xylazine were purchased from Barber Veterinary Supply (Lynchburg, VA). 125I-labeled bovine serum albumin (BSA) was from ICN Radiopharmaceuticals (Irvine, CA).

Preparation of 125I-ZM241385

ZM241385 (10 nM) was radioiodinated with carrier-free \( ^{125}\text{I} \) (2200 Ci/mM) by adding chloramine T (10 μL of 1 mg/mL in water) to 1–2 mCi NaI (2200 Ci/mM) and 10 μL of ZM241385 (1 mM in methanol). After incubation for 5–10 min at room temperature, the reaction was quenched by adding 50 μL of aqueous sodium metabisulfite (20 mg/mL) [32]. We separated product the reactants by high-performance liquid chromatography (HPLC) over a C18 column using 5 mM phosphate buffer, pH 6.0, and an isopropanol gradient from 30% to 100% over 30 min at a flow rate of 0.5 mL/min (figure 1). 125I-ZM241385 was detected by use...
of an in-line gamma detector; UV absorbance was monitored at 255 nm. The product was stored at –20°C until use.

**Human PMNL Purification**

Human PMNL were purified from normal heparinized (10 U/mL) venous blood by a one-step ficoll-hyphae separation procedure [35] (>98% PMNL; >95% viable as determined by trypan blue exclusion) containing <50 pg/mL LPS). After separation, the PMNL were washed with HBSS 3 times.

**Recombinant Human Adenosine Receptor Binding Assay**

To evaluate the potency and selectivity of ZM241385 [36, 37], on human adenosine receptors, the four subtypes of recombinant human adenosine receptors were stably expressed in HEK-293 cells (American Type Culture Collection [ATCC], Manassas, VA) or CHO-K1 (ATCC) cells. The dissociation constants, summarized in table 1, were determined by competition for radioligand binding to membranes derived from the transfected cells [34, 38].

**Adenosine A<sub>2A</sub> Receptors on Human PMNL**

Binding of 125I-ZM 241385 to intact human PMNL was assayed. Neutrophils (0.9 × 10<sup>6</sup>) were suspended in 0.1 mL of PBS, mixed 1 : 1 with radioligand, and incubated for 2 h at room temperature. Receptor density and affinity were calculated as described elsewhere [38].

**Human PMNL [cAMP] and PMNL Adherence to a Biologic Surface**

A 24-well tissue culture plate was coated with human fibrinogen (5 mg/mL in 1.5% sodium bicarbonate; 0.5 mL/well; Sigma) overnight at 37°C in 5% CO<sub>2</sub>. PMNL (3–4 × 10<sup>5</sup>/0.5 mL/sample) were incubated within a well of the coated plate for 45 min in 0.5 mL of HBSS containing 0.1% HSA and ADA (1 U/mL) in the presence and absence of recombinant human TNF-α (10 U/mL), WRC-0470 (3–300 nM), and rolipram (100 nM) in tissue culture wells that had been coated overnight with human fibrinogen (see above; modified from [14]). The supernatants were placed on ice and centrifuged (2000 g for 10 min) to remove any remaining suspended cells, and the optical density (OD) of the supernatants was measured at 550 nm against matched SOD (200 U/mL)-containing samples. SOD-inhibitable superoxide released in 90 min (nanomoles) was calculated from the extinction coefficient for cytochrome c [41].

**PMNL Oxidative Activity (Human Whole Blood)**

Heparinized human whole blood (0.8 mL) was incubated at 37°C (30 min) with ADA (1 U/mL) and catalase (0.062 mg/mL) in the presence and absence of recombinant human TNF-α (1 U/mL), WRC-0470 (3–300 nM), and rolipram (100 nM) in tissue culture wells that had been coated overnight with human fibrinogen (see above). The OD of the samples was read at 550 nm against matched SOD (200 U/mL) samples at 30-min intervals by an ELISA plate reader (Titertek, McLean, VA). We calculated the amount of SOD-inhibitable superoxide (nanomoles) released in 0–150 min [41].

### Table 1. Dissociation constants of ZM241385 for recombinant human adenosine receptor subtypes.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Radioligand&lt;sup&gt;a&lt;/sup&gt;</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A&lt;sub&gt;2A&lt;/sub&gt; selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>125I-ABA</td>
<td>250 ± 89</td>
<td>132</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>125I-ZM241385</td>
<td>1.9 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>A&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>125I-ABOPX</td>
<td>33 ± 10</td>
<td>17</td>
</tr>
<tr>
<td>A&lt;sub&gt;3&lt;/sub&gt;</td>
<td>125I-ABA</td>
<td>270 ± 91</td>
<td>142</td>
</tr>
</tbody>
</table>

<sup>a</sup> 125I-ABA, N6-(4-amino-3-[125I]iodobenzyl)adenosine, 125I-ABOPX, 3-(4-aminoo-3-[125I]iodobenzyl)-8-phenyl-4-oxyacetate-1-propyl-xanthine; 125I-ZM241385, 4-[2(7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino)ethyl]-3-[125I]iodophenol.

<sup>b</sup> Mean ± SE, 3 experiments.

**Dose-response data.** PMNL (10<sup>6</sup>/mL) were incubated for 90 min in 1 mL of HBSS containing 0.1% HSA, cytochrome c (120 μM), and catalase (0.062 mg/mL) in the presence and absence of recombinant human TNF-α (1 U/mL), WRC-0470 (3–300 nM), and rolipram (100 nM) in tissue culture wells that had been coated overnight with human fibrinogen (see above; modified from [42]). The primed blood samples were stimulated with or without FMLP (1 μM; 15 min), placed on ice, and red blood cells were lysed with FACs lysing solution (Becton Dickinson), washed, and the leukocytes were resuspended in PBS. These samples containing mixed leukocytes were gated for PMNL by forward and side scatter, and the fluorescence of 10,000 PMNL was measured in the FL1 channel of a fluorescence-activated cell sorter (FACScan; Becton Dickinson). The results are reported as relative mean fluorescence intensity.
Human PMNL Degranulation (Adherent Cells)

PMNL (3 × 10⁶/mL) were incubated for 120 min in 1 mL of HBSS containing 0.1% HSA, with or without recombinant human TNF-α (10 U/mL), WRC-0470 (3-300 nM), and rolipram (300 nM) in a tissue culture well that had been coated overnight with human fibrinogen (see above; modified from [43]). The supernatant fluids with any suspended PMNL were harvested after incubation and centrifuged (2000 g for 10 min) to remove suspended cells, and the cell-free supernatants were frozen. Release of lysozyme, a component of PMNL primary and secondary granules, was assayed. Lysis of a suspension of *M. lysodeikticus* by the “cell-free supernatant” was measured by spectrophotometric analysis (540 nm) to determine how much granule contents was released to the surrounding medium [44].

Rat Bacterial Meningitis Model

We used a rat model of bacterial meningitis extensively characterized in our laboratory [30]. Adult Wistar rats (Hilltop Lab Animals, Scottdale, PA; Harlan Sprague-Dawley, Indianapolis; ~175-195 g) were anesthetized with intramuscular injections of ketamine (75 mg/kg) and xylazine (5 mg/kg). Bacterial meningitis was induced via intracisternal inoculation of LPS (200 ng) derived from *E. coli* 026:B6. The animals were then infused with rolipram, WRC-0470, or both over the duration of the experiment with a Harvard pump. We sampled CSF and blood 4 h after inoculation and determined alterations in BBBP and white blood cell (WBC) counts. CSF WBC concentrations were determined by standard hemocytometer methods. For assessment of BBBP, rats were given an intravenous injection of 5 µCi of ¹²⁵I-labeled BSA concomitant with intracisternal inoculation. Equal samples of CSF and blood were counted in a gamma counter, and after subtraction of background radioactivity, the percentage of BBBP was calculated by the following formula: \( \%\text{BBBP} = (\text{cpm CSF/cpm blood}) \times 100 \).

WRC-0470 Concentrations in Rat Serum Samples

The high affinity binding of WRC-0470 to recombinant A₂A receptors was used to develop a sensitive radioreceptor assay to detect low (<1 µM) levels of the compound in plasma that cannot be detected by HPLC. Because compounds in plasma interfere with radioligand binding, the plasma was first applied to 1 × 2 cm C18 Seppaks, washed with methanol/water (1:9), eluted with 100% methanol, dried in a SpeedVac (Savant, Holbrook, NY), and reconstituted in PBS. This procedure eliminated interference from plasma. When rat plasma was spiked with 10 µM WRC-0470, 7.6 µM was recovered in the assay.

Statistical Analysis

Results are expressed as mean ± SE. Significance (*P* < .05) was determined by Student’s two-tailed *t* test. In vivo data were analyzed by one-way analysis of variance (ANOVA), Kruskal-Wallis nonparametric ANOVA test, and the Mann-Whitney test (Mac-Instant Statistics; GraphPad Software; San Diego). Data are typical of two or more replicate experiments.

Results

Adenosine A₂A receptors on PMNL. By using ¹²⁵I-ZM241385, we characterized adenosine A₂A receptors on intact
PMNL. The $B_{max}$ is 1.4 fmol/10^6 PMNL (843 receptors/cell) and the $K_D$ is 0.125 nM (figure 2). By examining all four recombinant human adenosine receptor subtypes, we found that ZM241385 is 17- to 142-fold selective for the human A$_{2A}$ adenosine receptor compared with the other receptor subtypes (table 1).

PMNL [cAMP] and adherence. TNF (10 U/mL) markedly increased PMNL adherence to fibrinogen at 45 min (220% of unstimulated PMNL). When added at 300 nM, neither WRC-0470 nor rolipram significantly affected PMNL adherence, but TNF-enhanced adherence was decreased by WRC-0470 (300 nM) plus rolipram (300 nM; $P = .014$). We did not observe an effect of TNF (10 U/mL) on adhering PMNL [cAMP] (data not shown). PMNL [cAMP] was inversely correlated with cell adherence. We observed a corresponding increase in [cAMP] with the decrease in adherence in the WRC-0470 plus rolipram treatment group. WRC-0470 (300 nM) plus rolipram (300 nM) synergistically increased TNF-stimulated adhering PMNL [cAMP] to 138% of control ($P = .012$). ZM241385 (100 nM) blocked the effects of WRC-0470 plus rolipram on PMNL adherence and [cAMP] (figure 3).

PMNL superoxide release (adherent cells). Neither WRC-0470 (30–300 nM) nor rolipram (100 nM) by themselves had a significant effect on TNF-α-stimulated adhering PMNL superoxide release measured at 90 min. In contrast, when combined with rolipram, WRC-0470 synergistically decreased TNF-α-stimulated adherent PMNL oxidative activity ($P < .050$; figure 4). TNF-α stimulated a prolonged oxidative burst in PMNL adhering to a biologic surface (figure 5). WRC-0470 (300 nM) plus rolipram (300 nM) prevented the rise in superoxide release stimulated by TNF-α. The effect of WRC-0470 plus rolipram was completely counteracted by ZM241385 (100 nM; figure 5). The activities of WRC-0470 and rolipram cannot be attributed to nonspecific toxicity to the cells or to scavenging of superoxide, since the combination of WRC-0470 and rolipram does not inhibit superoxide release from PMNL stimulated with PMA (data not shown).

PMNL oxidative activity (whole blood). WRC-0470 (30

Figure 3. WRC-0470 (300 nM) with rolipram (300 nM) increases polymorphonuclear leukocyte (PMNL) [cAMP] and decreases recombinant tumor necrosis factor-α-stimulated PMNL adherence to fibrinogen-coated surface. Data are mean ± SE of 5 separate experiments. *$P < .05$, [cAMP] vs. control (no WRC-0470 or rolipram); **$P < .05$, adherence vs. control.

Figure 4. Synergy between A$_{2A}$ adenosine receptor selective agonist WRC-0470 and rolipram (100 nM) to inhibit tumor necrosis factor (TNF)-α-stimulated adhering polymorphonuclear leukocyte (PMNL) oxidative activity. Mean ± SE of 5 experiments (control TNF-α-stimulated activity, 18.2 ± 1.0 nmol/90 min/10^6 PMNL/mL; *$P < .05$ vs. TNF-α-stimulated control PMNL). WRC-0470 (○); WRC-0470 + rolipram (▲).
nM) decreased PMNL oxidative activity and acted synergistically with rolipram (300 nM) in human whole blood samples stimulated with FMLP (1 μM). In whole blood samples primed with TNF-α (10 U/mL) and then stimulated with FMLP (1 μM), oxidative activity was decreased by the combination of WRC-0470 (300 nM) and rolipram (300 nM) to levels lower than those observed in unprimed PMNL (figure 6). Hence, WRC-0470 and rolipram retain their synergistic action in whole blood.

**PMNL degranulation (adherent cells).** In addition to decreasing TNF-α-stimulated adherent PMNL oxidative activity, WRC-0470 combined with rolipram also decreased degranulation of activated PMNL adhering to a biologic surface. WRC-0470 (300 nM) or rolipram (300 nM) had little activity alone but synergistically decreased degranulation (P = .027; table 2).

**Rat bacterial meningitis model.** WRC-0470 was measured in samples extracted from rat blood by a bioassay. Figure 7 shows a standard curve from which the values in plasma of rats infused with WRC-0470 were derived. A steady state plasma concentration of 0.8 ± 0.17 μM (n = 2) was reached in animals infused at 0.300 μg/kg/h, and a plasma level of 2.33 ± 0.29 μM (n = 4) was obtained at an infusion rate of 0.6 μg/kg/h.

The dramatic and potent effect of infusing WRC-0470 on leukocyte pleocytosis can be seen in figure 8A. Infusion of WRC-0470 at 0–0.9 μg/kg/h caused a dose-dependent inhibition of pleocytosis into the subarachnoid space with 95% inhibition seen during WRC-0470 administration at 0.9 μg/kg/h (P < .05 vs. control). The effect WRC-0470 had on BBBP is illustrated in figure 8A. Significant responses were seen in a dose range of 0.6–0.9 μg/kg/h (P < .05 vs. control).

Figure 8B illustrates the effect of rolipram on CSF pleocytosis. Rolipram caused a dose-dependent inhibition of pleocytosis in a range of 0–0.01 μg/kg/h, with 0.01 μg/kg/h inhibiting 99% of the pleocytosis (P < .05). Rolipram at either 0.01 or 0.005 μg/kg/h showed significant inhibition of LPS-induced increase in BBBP (P < .05), whereas a dose of 0.002 μg/kg/h had no significant effect (figure 8B).

The effect of a combination of rolipram and WRC-0470 on CSF pleocytosis is illustrated in figure 8C. Rolipram (0.001 μg/kg/h) in combination with WRC-0470 (0.1 μg/kg/h) inhibited migration of leukocytes (200 ± 70 WBC/μL) into the subarachnoid space to a greater extent than rolipram (1670 ± 1473 WBC/μL; P < .05) or WRC-0470 (600 ± 308 WBC/μL; P < .05) alone.

![Figure 5](image-url)  
**Figure 5.** Effect of WRC-0470 (300 nM), rolipram (300 nM), and ZM241385 on superoxide release from adhering polymorphonuclear leukocytes (PMNL). Release of superoxide was measured from PMNL adhering to fibrinogen surface. Each data point is mean of 3–5 separate experiments. *P < .05 vs. no ZM241385.

![Figure 6](image-url)  
**Figure 6.** WRC-0470 decreases oxidative activity of tumor necrosis factor-α-primed FMLP-stimulated polymorphonuclear leukocytes in human whole blood and acts synergistically with rolipram. Data are mean ± SE of 5–10 separate experiments. *P < .05 vs. activity in absence of WRC-0470 and rolipram.
Table 2. WRC-0470 with rolipram synergistically decreases tumor necrosis factor (TNF-α)-stimulated adherent polymorphonuclear leukocyte (PMNL) degranulation.

<table>
<thead>
<tr>
<th>PMNL stimulated with</th>
<th>ng/mL lysozyme released/3 x 10⁶ PMNL/mL/120 min³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>430 ± 100</td>
</tr>
<tr>
<td>TNF-α and WRC-0470 (300 nM)</td>
<td>360 ± 74</td>
</tr>
<tr>
<td>TNF-α and rolipram (300 nM)</td>
<td>400 ± 140</td>
</tr>
<tr>
<td>TNF-α and WRC-0470 (300 nM) + rolipram (300 nM)</td>
<td>140 ± 41</td>
</tr>
</tbody>
</table>

a Mean ± SE, 6 experiments.

Discussion

Radioligand binding and functional assays suggest that human PMNL have both A₁ and A₂A receptors [3–5]. In addition, by reverse transcriptase polymerase chain reaction there is evidence of mRNA for both A₂B [45] and A₃ [20] receptors in human neutrophils. We believe the current study is the first to utilize a highly selective antagonist radioligand to measure the number and affinity of adenosine A₂A receptors on intact human PMNL. ZM241385 binds to human PMNL with very high affinity (nM). ¹²⁵I-ZM241385 binds with 10 times higher affinity than the agonist [³H]-CGS 21680 that has been used as a probe for PMNL A₂A receptors in previous studies [5].

Our finding are summarized in figure 9. A selective adenosine A₂A receptor agonist (WRC-0470) increases PMNL [cAMP], and decreases TNF-α and FMLP-stimulated PMNL adherence, oxidative activity, and degranulation. A highly selective A₂A adenosine receptor antagonist (ZM241385) blocks both WRC-0470 stimulation of PMNL [cAMP] and counteracts the effects of WRC-0470 to inhibit PMNL inflammatory functions. In contrast, inhibition of cAMP conversion to 5'-AMP by a type IV phosphodiesterase inhibitor (rolipram) synergistically enhances WRC-0470-stimulated PMNL [cAMP], and augments the effects of WRC-0470 on PMNL adherence, oxidative activity, and degranulation.

In 1983, adenosine was shown to inhibit the production of reactive oxygen species from suspended PMNL stimulated with certain stimuli [9]. In suspended PMNL, TNF is not a strong stimulus to evoke an oxidative burst but will prime nonadherent cells to produce an increased oxidative burst in response to a second stimulus [46]. Adenosine, by binding to A₂A receptors, can markedly decrease the primed response of nonadherent PMNL by acting on the second response rather than on the priming step [14, 16, 47]. In contrast to the response of nonadherent PMNL, TNF by itself elicits a slow but large and prolonged activation of PMNL adhering to a biologic surface and decreases [cAMP] in the adhering PMNL [48, 49]. High concentrations of TNF are required to decrease adherent PMNL [cAMP], [49]. At the concentration used in the present experiments (10 U/mL), TNF has no effect on adherent PMNL [cAMP].

Adenosine (IC₅₀ ≈ 500 nM) and other compounds that increase PMNL [cAMP], inhibit TNF-stimulated adherent human PMNL oxidative activity when the PMNL are adhered to a biologic surface [11, 49]. The results of the present study indicate that the adherent PMNL oxidative response is decreased by A₂A adenosine receptor activation. The concentrations of WRC-0470 that are active are similar to plasma levels detected in vivo in rats treated with WRC-0470 that effectively inhibit pleocytosis.

Because agonist binding to adenosine A₂A receptors can stimulate cAMP synthesis within cells and increased [cAMP], has an antiinflammatory effect [21, 50, 51], we measured the effects of WRC-0470 both in the absence and presence of a type IV cAMP PDE inhibitor [24]). We noted previously that in TNF-primed FMLP-stimulated suspended PMNL [14] there is marked synergy between a selective adenosine A₂A receptor agonist and rolipram to inhibit PMNL oxidative activity. In the present study, synergy was detected in both whole blood and in the oxidative burst of TNF-stimulated adherent PMNL. We also observed synergy between an A₂A adenosine agonist and a type IV PDE inhibitor when measuring TNF-stimulated PMNL adherence (which correlated with PMNL [cAMP]) and degranulation of adherent PMNL. These data suggest that adenosine activity may be mediated by [cAMP], and thus type IV PDE inhibition is a potential viable strategy for potentiating possible therapeutic antiinflammatory effects of agonists of A₂A adenosine receptors.

Increased PMNL [cAMP], inhibits PMNL spreading on biologic surfaces and reorganization of the cytoskeleton stimulated by proinflammatory stimuli in adherent PMNL [52]. The an-

Figure 7. Radioreceptor assay of WRC-0470 in rat plasma. Standards and samples were purified as described in Materials and Methods. Standard curve is fitted to 4-parameter logistic equation.
Figure 8. Effects of WRC-0470 and rolipram on lipopolysaccharide-stimulated white blood cell pleocytosis and blood-brain barrier permeability in rat bacterial meningitis model. A–C, with and without WRC-0470 (A) (0–0.9 μg/kg/h), rolipram (B) (0–0.01 μg/kg/h), WRC-0470 (0.1 μg/kg/h), and rolipram (C) (0.001 μg/kg/h). Mean ± SD, n = 2 rats for each point. CSF, cerebrospinal fluid.

The inflammatory effects of increased [cAMP], may be mediated by protein kinase A phosphorylation of small GTP-binding proteins that control cytoskeletal organization and the assembly of the neutrophil NADPH oxidase. The effect could be through phosphorylation of Rap1A, preventing incorporation of cytochrome b₅₅₈ into the oxidase complex [53] or by effects on RhoA [54–56] resulting in diminished phospholipase D activity [57] and decreased adherent PMNL oxidative activity [58].

We chose the rat bacterial meningitis model to investigate the effect of an A₂A receptor agonist to inhibit an inflammatory response in vivo. Adenosine has effects other than those on PMNL function that may play a role in the inflammatory response to endotoxin challenge. For example, agonist binding to adenosine A₂A receptors [23, 59–61] and/or inhibition of type IV PDEs [22, 62] results in decreased monocyte/macrophage production of TNF-α in response to LPS stimulation. Recently, we observed that binding of the selective A₂A agonist (CGS 21680; 100–1000 nM) decreases LPS-stimulated TNF-α pro-
Figure 9. WRC-0470 inhibition of neutrophil (PMNL) activation. WRC-0470 binds selectively to adenosine A2A receptors on PMNL. A2A receptors are coupled through G protein, Gs, to adenylyl cyclase, which when activated converts ATP to cAMP. ZM241385 is a selective competitive antagonist at adenosine A2A receptors that block WRC-0470 binding to these receptors. Rolipram by inhibiting type IV phosphodiesterase breakdown of cAMP to 5’AMP augments PMNL [cAMP] accumulation stimulated by WRC-0470. High cellular [cAMP] decreases tumor necrosis factor (TNF-α) and FMLP (TNF-α/FMLP)-stimulated PMNL adherence, reactive oxygen species production, and degranulation.

Even though the immune system is essential for combating invading pathogens, numerous disease processes are caused or exacerbated by inappropriate activation of the immune system, resulting in inflammation, debilitating tissue damage (e.g., arthritis), and sometimes death (e.g., bacterial meningitis or septic shock) [64]. All of the effects seen with WRC-0470 and rolipram were observed at infusion rates 10²- to 10³-fold lower than dosages of A2A selective agonists required to induce hemodynamic responses (tachycardia, lowered blood pressure) [26] or of rolipram to cause emesis [27] in the rat. Thus, the powerful antiinflammatory actions of specific A2A adenosine receptor agonist stimulation combined with type IV PDE inhibition are encouraging in suggesting a feasible therapeutic strategy for treating these disease processes while minimizing adverse side effects. Further studies will be necessary to define conditions for optimal synergy between these two classes of antiinflammatory compounds.

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